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Detection of Hepatitis C Virus Core Protein Circulating Within Different Virus Particle Populations

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Progress in studying pathogenesis and increasing the reliability of hepatitis C diagnosis can be achieved by analysis of different forms of virus particles circulating in blood of both patients and infected persons. Detection of hepatitis C virus (HCV) proteins faces two basic difficulties: low concentration of HCV proteins, and their blocking by antibodies. The aim of this work was to develop a method for the detection of nucleocapsid (core) protein in the plasma of HCV-infected persons using monoclonal antibodies (MABs). Twenty-seven anti-HCV-positive donor plasmas were studied of which 21 contained HCV RNA and 6 were negative. The plasmas were centrifuged for 3 hr at 143,000 g and the antigenic activity of core-protein was studied in the pellets by EIA using four MABs able to recognize four nonoverlapping determinants, two at N-terminus and two at C-terminus of recombinant core (1–150 aa). The determinants detected were present in the natural core protein of at least two genotypes (1b and 3a). Maximal efficiency of recombinant protein detection was achieved with 2 MABs, whereas a combination of 4 MABs was necessary for optimal detection of natural core protein. This is indicative of different conformational structures of natural protein and its gene-engineered analog. The sensitivity of core detection by monoclonal sandwich assay was 1 ng/ml in the pellet or 5 pg/ml after normalization to the initial plasma volume. To dissociate immune complexes, the pellet was treated with 2.5 M KBr after first treating the pellet with the nonionic detergent Tween 80 to remove the virus lipid envelope. Using this treatment protocol, core protein was found in 19 of 21 RNA positive plasmas. ***J. Med. Virol. 55:1–6, 1998.***

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INTRODUCTION

The genome of hepatitis C virus (HCV) encodes several structural and nonstructural proteins (core, E1, E2/NS1, NS2–NS5) the functions of which are not completely known, since substantial amounts of viral material is required for virus visualization and characterization using conventional techniques. Many attempts to cultivate HCV in vitro have been undertaken [Carloni et al., 1993; Cox et al., 1996; Ito et al., 1996] using plasma from HCV-infected patients as the source.

Hepatitis C diagnosis is based mainly on detection of specific antibodies to HCV proteins. However, data from many laboratories suggest that antibody testing alone is not sufficient to detect all HCV-positive specimens, especially in primary HCV infections when antibody appearance may be delayed, and in some cases significantly delayed [Schmilovitzweiss et al., 1993]. Furthermore, up to 10% of chronic patients with pronounced liver disease remain seronegative [Trepo et al., 1993]. The number of such patients is higher under immune deficiency conditions (caused by organ and tissue transplantation or HIV infection). Additional methods of virus detection, such as detection of HCV RNA by PCR and detection of viral proteins in the liver sections by immunohistochemical staining were applied, and such nonroutine tests are becoming more widely used in the laboratory diagnosis of HCV-infection. The absence of a test suitable for HCV-protein determination in blood has provided the impetus for this investigation—namely, to develop a method for detection of the relatively genetically stable HCV core protein in the plasma using monoclonal antibodies. This technique could be useful for monitoring different stages of virus

infection and for making judgments about disease pathogenesis.

Two difficulties had to be overcome: low core concentrations in the plasma, and its blocking by antibodies present in immune complexes. We now report the successful detection of HCV core protein using a monoclonal antibody sandwich enzyme immunoassay (EIA) after pelleting and disruption of immune complexes.

MATERIALS AND METHODS

Recombinant Proteins

A full-sized recombinant protein (R191) containing all amino acids residues (aa) of the predicted full length core protein was expressed in *E. coli* as described earlier [Khudyakov et al., 1993]. Recombinant protein (R150) containing 1-150 N-terminal aa residues from the core sequence was expressed in *E. coli,* and purified by ion-exchange chromatography on phosphocellulose [Masalova et al., 1996].

Seven recombinant proteins were expressed in *E. coli* from DNA-constructs as hybrids with β-galactosidase having the following core aa sequences: 1-160, 1-80, 1-25, 19-31, 33-56, 55-72, and 58-84 [Masalova et al., 1996].

Human Plasmas

The plasmas (n = 34) were obtained from the Blood Transfusion Station at the Hematology Center (Moscow) from altruistic donors. Anti-HCV antibodies were detected in 27 plasmas by the Ortho HCV 3.0 ELISA Test System, whereas 7 plasmas were anti-HCV–negative. In addition, human antibodies to the core protein were detected by an indirect EIA using R150 at a concentration of 0.5 ug/ml adsorbed to the solid-phase.

Monoclonal Antibodies (MABs)

Balb/c mice were immunized intraperitoneally 4 times at monthly intervals with 20 ug of purified R150. Cell hybridization was carried out according to the method of Kohler and Milstein [1975]. MABs screening was done by an indirect solid-phase EIA. For solid-phase sensitization the following antigen concentrations were used: R150, 0.5 ug/ml; *E. coli* lysate, 10 ug/ml; β-galactosidase (Sigma), 10 ug/ml; recombinant proteins—fragments of core, 50 ug/ml. MABs were isolated from the ascitic fluids by double precipitation with ammonium sulfate (50% saturation) and purified by affinity chromatography on a Protein A-Sepharose CL-4B column (Pharmacia). The protein concentration was determined by the method of Bradford [1976]. MABs subtype and light chain type determination were done by EIA using the Mouse-Hybridoma-Subtyping Kit (Boehringer Mannheim). Purified MAB conjugation with horseradish peroxidase was carried out by the modified periodate method [Nakane and Kawaoi, 1974]. The constants of MAB binding with R150 were estimated by EIA and the results processed by Scatchard's method [Frankel and Gerhard, 1979].

Competitive Analysis

To determine the MABs epitope specificity, reciprocal competitive EIA was carried out. The MAB conjugates were incubated with different concentrations (100, 20, 4, 1, and 0 ug/ml) of purified nonlabeled antibodies in the plate wells sensitized with R150. The results were expressed as the extent of competition between different MABs for binding with R150 antigen.

Competition between antigens for binding with MABs was determined by EIA. Recombinant core protein fragments at concentrations of 500, 100, 20, 4, and 0 ug/ml were incubated with MABs and then transfered to wells sensitized with R150, and MABs binding to R150 were measured.

Immunoblot and Immunodot

For immunoblot analysis proteins were separated by electrophoresis in 12% SDS PAGE in the presence of molecular mass markers (Pharmacia), and the separated products were transfered to nitrocellulose (Schleicher & Schuell, 0.45 um) by electroelution. For each immunodot probe 1 ug of protein was applied onto the nitrocellulose. Immune staining of nitrocellulose strips was done using a standard technique.

Preparation of Plasma for Detection of HCV Core Antigen

Individual donor blood plasmas (60 ml) were clarified by centrifugation (3,500 g, 15 min) in K23D centrifuge (Janetzki, Germany) and then ultracentrifuged (143,000 g, 3 h, 4°C) in Sorvall ultracentrifuge OTD 65 (Du Pont, USA). The pellets were resuspended in a minimal volume of 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The pellets underwent different treatments to disintegrate immune complexes and to solubilize the lipid envelope of the virus. The treatments included detergents and denaturing agents (Tween-80, NP-40, SDS, Triton X-100, 2 ME, Urea), chaotropic agents (KBr, KJ, NaCl, and KSCN, 2–2.5 M each), high and low pH values (DMSO, pH 9.5; glycine, pH 9.5; ethylene glycol, pH 9.5; HCl, pH 2.0; glycine, pH 3.0), as well as various combinations.

EIA for the Detection of HCV Core Antigen

The HCV core-protein antigen was detected by an EIA sandwich variant. The first antibodies were adsorbed to the microtiter well at a concentration of 10 ug/ml (0.05 M carbonate-bicarbonate buffer, pH 9.5), followed by a blocking buffer (PBS, pH 7.4, 0.1% Tween-20, 10% human blood serum) for 1 hr at room temperature. After incubation with the sample (2 hr, 37°C) and washing, the second antibody (MAB conjugates with peroxidase) diluted with blocking buffer was added and incubated for 1 hr at 37°C. Following a wash step the substrate was added upon washing and after 20 min of incubation in the dark the reaction was stopped by the addition of 2M H_2SO_4. Absorbtion was measured at 492 nm. A cutoff was determined as twice

TABLE I. Properties of the Monoclonal Antibodies to Recombinant HCV Core Antigen

MAB	Ig class, type of light chains	Association constant (M^{-1})	EIA titres with R150 (dilutions)	Epitope specificity (aa residues)
27	IgG1, κ	$3{,}6 \times 10^9$	5×10^{-6}	1–80
37	IgG1, κ	$4{,}3 \times 10^8$	10^{-5}	1–80
D4	IgG1, κ	$6{,}0 \times 10^9$	10^{-6}	80–150
G7	IgG2b, κ	$2{,}0 \times 10^9$	10^{-6}	80–150

the average of 3 of the negative control optical density values (anti-HCV and HCV RNA–negative).

Detection of HCV RNA

HCV RNA was detected by nested reverse transcriptase polymerase chain reaction (RT-PCR) using synthetic primers from the 5′-nontranslated region of the HCV genome [Okamoto et al., 1990]. In some specimens HCV genotypes were also determined according to Okamoto et al. [1993].

RESULTS

Characterization of Monoclonal Antibodies

Four clones exhibiting the highest affinity to R150 were selected. The MAB properties are described in detail elsewhere [Masalova et al., 1996]. A short summary of MABs characteristics is given in Table I.

All hybridomas produced class G immunoglobulins and were characterized by a high affinity constant. The specificity of MABs was confirmed by immunoblotting. It was shown earlier that the molecular mass of the full-size core protein R191 was 27–28 kDa, and that of the recombinant protein R150 was 25 kDa [Khudyakov et al., 1993; Masalova et al., 1996]. Each MAB reacted with the full-sized recombinant core protein (R191) and stained a 28 kDa protein zone (major band) and a 23 kDa species (minor band). When the purified core R150 preparation was used all MABs reacted with a protein in the 25 kDa (major band) and 18 kDa (minor band) regions (data not shown). Lower than expected molecular weight bands are evidently caused by MAB interaction with the products of recombinant protein processing or proteolysis. None of the clones reacted with the *E. coli* lysate proteins present in control preparation.

To determine the MABs epitope specificity we have cloned several immunoreactive fragments of different lengths from the core region which overlap the 160 N-terminal aa. Solid-phase EIA, immunodot, and competitive EIA using these fragments have shown that MABs recognize four nonoverlapping epitopes. As shown in Table I, two of these epitopes are localized within 1-80 aa, and two others are within the 80-150 aa region of the core protein [Masalova et al., 1996].

EIA Sandwich Variant for the Detection of HCV Core Antigen

The ability of MABs to detect recombinant core antigenic activity by EIA is shown in Table II. A combination of MAB D4 as a solid-phase reagent with MAB 27 as a conjugate with peroxidase exhibited the highest sensitivity (up to 1 ng/ml) compared to other sandwich variants. A MAB mixture on the solid phase did not result in any advantage in sensitivity.

TABLE II. The Dependence of the Recombinant Core EIA Detection Sensitivity on the Sandwich Composition

MABs on the solid phase	Peroxidase conjugates of MABs		
	27	37	D4
27	100[a]	25	6
37	12	250	12
D4	1	12	100
G7	5	25	30
Mixture of the MABs	4	12	6

[a]ng/ml of purified R150.

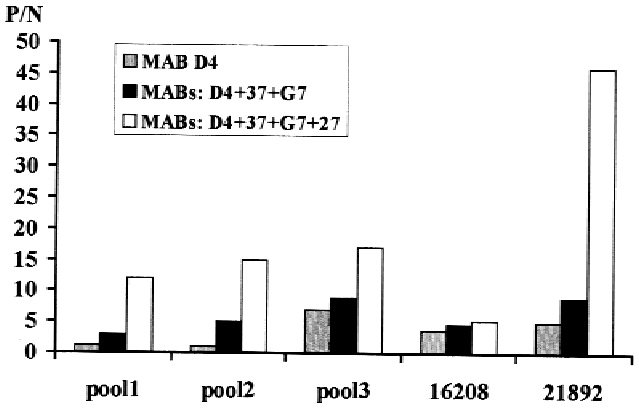

Fig. 1. Efficiency of different solid-phase monoclonal antibodies to detect HCV-core protein by an EIA sandwich variant. Pool, 1–3 represent pools of 4–5 donor plasmas. Samples 16208 and 21892 are individual plasma donations.

Our further investigations were focused on the detection of natural core protein using a MABs EIA. Donor plasmas seropositive to the R150 protein were selected. The experiments were carried out on two individual plasma samples (16208, 21892) and two pools of 4 to 5 plasma donors each. Donor plasmas free of anti-HCV antibodies (5 samples) served as negative controls. The plasmas were clarified and ultracentrifuged and the pellets were collected and analyzed by EIA for core protein content. The results were estimated as the signal-to-background absorption ratio (P/N) (Fig. 1).

At first, a two-site sandwich exhibiting the highest sensitivity for recombinant core detection was chosen: MAB D4 on the solid-phase, and MAB 27 as conjugate. However, our experiments as shown in Table II demonstrated that the sensitivity for natural core detection increased 2 to 5 times when the solid-phase was sensitized with a D4, 37, and G7 mixture. It increased further when MAB 27 was added to the mixture. Replacement of the MAB27 conjugate (second antibodies) by conjugates of different MABs or various combinations did not increase the analytical sensitivity (data not shown).

TABLE III. Detection of HCV core antigen activity and of HCV RNA in anti-HCV antibody-positive plasmas

Group of plasmas	No. plasma	EIA titer with R150, $\times 10^{-3}$	HCV-RNA (genotype)	Concentration of core in pellets (EIA), ng/ml			Concentration of core in plasmas (pg/ml)
					After treatment		
				Untreated	Tween-80	KBr + Tween-80	
I	1–6	1,6	–	0[a]	0[a]	0[a]	0[b]
II	22822	3,2	+ (1b)	0	0	0	0
	22817	12,0	+ (1b)	0	0	0	0
III	16249	1,0	+ (3a)	0	16	0	160
	20841	0,8	+	0	2	0	10
	17083	1,0	+	0	1	0	5
IV	32839	1,0	+ (3a)	0	3	4	40
	46066	12,0	+ (1b)	0	15	25	250
	14893	12,0	+ (1b)	0	21	30	300
V	39794	1,0	+	0	0	5	25
	22811	3,2	+ (1b)	0	0	8	80
	16208	3,2	+ (1b)	0	0	30	150
	32789	6,4	+ (1b)	0	0	45	450
	39635	3,2	+	0	0	90	450
VI	20300	1,0	+	10	11	0	330
	15197	0,8	+ (1b)	14	10	0	140
	35341	12,0	+	15	12	0	150
	30000	6,4	+ (1b)	27	21	9	270
VII	22722	3,2	+ (1b)	5	5	8	80
	12594	3,2	+	10	12	28	280
	21892	3,2	+ (1b)	32	30	53	790
	50000	12,0	+ (1b)	43	45	85	850

[a]<1 ng/ml core in pellet.
[b]<5 pg/ml core in plasma.

Detection of Core Protein Within Immune Complexes

Pellets obtained by ultracentrifugation may contain both HCV and anti-HCV antibodies. Experiments have shown that anti-core activity in the pellets approximated 20 to 75% of initial levels found in plasma. Since these antibodies could interfere with core protein detection, we have tested approximately 20 ways of immune complex dissociation and core release.

The core antigenic activity before and after various treatments was measured simultaneously on three specimens: recombinant protein R150, a pool of 12 anti-HCV–positive donor plasma pellets, and 5 anti-HCV–negative plasma as negative controls.

Experiments on a model system using R150 (data not shown) have shown that core antigenic activity survives treatment with nonionic detergents, SDS in the presence of 2-mercaptoethanol, high NaCl concentrations in the presence of Tween 80 and NP 40, and acid hydrolysis. On the other hand, treatment with DMSO and ethylene glycol at pH 9.5, SDS in combination with nonionic detergents, KI, KSCN, and urea almost completely blocked the interaction of R150 with MABs.

A 4- to 8-fold increasing in EIA signal was observed following treatment of natural core with SDS with 2-mercaptoethanol, KBr, and NaCl in the presence of Tween-80. Some treatments resulted either in a 1.5- to 2-fold increase or a decrease in EIA signal. Some agents (NaCl + NP40; SDS + 2ME; SDS + Triton X-100; SDS + NP –40; HCl, pH 2.0, 90 min., RT; glycine, pH 3.0, 10 min., 70°C; DMSO, pH 9.5; glycine, pH 9.5) resulted in false-positive reactions in the negative control. A comparison of these data shows that the highest sensitivity for natural core detection in plasma was achieved after the pellets were treated with 2.5 M KBr in combination with the nonionic detergent Tween-80 at a concentration of 0.5% (data not shown).

Detection of HCV Core Antigen in Individual Plasmas

Twenty-seven anti-HCV–positive and 7 anti-HCV–negative plasmas were analyzed for anti-R150 antibody activity by indirect EIA and for HCV RNA content by RT-PCR. After plasma ultracentrifugation the core antigenic activity was studied. The core content in the pellets was estimated using a calibration curve for recombinant core that showed a linear relationship between optical density by EIA and protein concentration within the range from 1 to 130 ng/ml.

Three treatment variants were studied simultaneously: untreated pellets, pellets treated with Tween-80 only, and pellets treated with KBr in combination with Tween-80. The core content was normalized to 1 ml of plasma.

All anti-HCV–negative plasmas showed negative results for antibodies to R150, RNA, and core antigenic activity (data not shown). For anti-HCV–positive plasmas, the results suggested that the plasma units can be classified into 7 groups (Table III): I, core and HCV RNA were not found; II, HCV RNA was positive, core was not found; III, core antigen activity was detected only after Tween-80 treatment; IV, core antigen activ-

ity was detected after Tween-80 treatment; after immune complex dissociation by KBr, nearly the same amount of protein was detected); V, a positive signal for core antigen activity was obtained by EIA only after the pellet was treated with KBr in the presence of Tween-80; VI, core protein was detected in untreated pellets; treatment with Tween-80 had no effect on the amount of detected protein, whereas the detergent affect in the presence of a high KBr concentration sharply decreased it or completely blocked detection; VII, core antigen was found both in untreated pellets and in those treated with Tween-80; the combined effect of KBr and detergent significantly increased detection.

Maximal core amount found in one sample was 850 pg/ml of plasma (Group VII), whereas the minimal amount detected was 5 pg/ml (Group III).

DISCUSSION

Published data show that the 50–70 N-terminal amino acids of the HCV-core protein exhibit the highest antigenicity [Akatsuka et al. 1993; Cerino et al., 1993; Nakagiri and Ichihara, 1995; Sallberg et al., 1994]. Some authors suggest that the C-terminal 70–191 aa is relatively insignificant for a humoral immune response [Goeser et al., 1994], while others report on the antigenicity of other core regions [Akatsuka et al., 1993; Sallberg et al., 1994]. The MABs obtained in this study recognize 4 individual nucleocapsid epitopes, 2 of which are localized within the region of 1–80 aa (MABs 27 and 37) and 2 others within 80–150 aa (MABs D4 and G7). Thus our results confirm other reports that antigenically active epitopes are located at both the N-terminus and C-terminus regions of HCV nucleocapsid protein.

It is known that some monoclonal antibodies to recombinant HCV proteins and to synthetic peptides do not recognize natural viral antigens [Gonzales-Peralta et al., 1994; Sansonno and Dammacco, 1993; Yap et al., 1994]. Interaction of MABs obtained in this study with natural HCV protein suggested that the antigenic determinants detected by them are characteristic of natural core of at least 2 HCV genotypes—1b, from which they were obtained [Khudyakov et al., 1993] and 3a (Table III). This observation suggests that these epitopes are conserved between 1b and 3a.

Maximal sensitivity for the detection of the recombinant protein was achieved with 2 MABs, whereas 4 were necessary for the optimum detection of natural protein. This observation suggests conformational differences between the natural core present in the plasma and its gene-engineered analog. It is important to note that the monoclonal sandwich EIA variant avoids the use of serum antibodies, which otherwise would result in instability of some properties [Biswas et al., 1994].

The presence of co-circulating anti-core antibodies activity, which would inhibit or block core detection, is a significant problem [Sansonno and Dammacco, 1993; Takahashi et al., 1992]. Treatment with high KBr concentrations in the presence of Tween-80 as indicated in this study makes possible immune complex dissociation exposing core antigen directly in the pellets obtained after a single cycle of plasma ultracentrifugation. This approach resulted in core protein detection in five samples of donor plasma (Table III, Group V), whereas without this treatment it was completely blocked by antibodies. At the same time, however, it has been shown that in some cases treatment with KBr + Tween-80 interferes with protein detection (Table III, Groups III and VI). This effect is evidently due to a decreased interaction between core and MABs at a high ionic strength created by KBr. The results of model experiments (data not shown) confirm this supposition.

Thus 2 EIAs of each pellet sample are necessary for reliable detection of core protein, one after treatment with Tween-80 and the other after treatment with a Tween-80-KBr-combination. The sensitivity of the proposed method is about 5 pg of core protein per ml of plasma. The maximal amount of core protein detected was 850 pg/ml, which correlates with the results of Takahashi et al. [1992] and Oshita et al. [1996], who obtained values of 670 pg/ml and 889 pg/ml, respectively.

Comparative analysis of core detected in nontreated pellets, pellets treated with a lipid solvent (Tween-80), and pellets treated with a substance to dissociate immune complexes (KBr in combination with Tween-80) suggests the nature of circulating HCV. For example, plasmas belonging to Group III may contain HCV virions free of immune complexes. Plasmas belonging to Group IV may contain both free and immune complexed virions. Because free virions were not detected, plasmas of Group V may contain core-antigen only within immune complexes. In 8 plasma units, nucleocapsids were found without detergent treatment (Groups VI and VII), suggesting that free nucleocapsid circulates without a lipid envelope. In Group VII, however, these free nucleocapsids were also found in immune complexes. No core protein was found in 2 RNA-positive plasmas of Group II, which is indicative of either the absence of viral genome expression or negligible core antigen concentration in these plasmas (less than 1 ng/ml in the pellets and less than 5 pg/ml in whole plasma).

It has been shown in recently published papers [Kanto et al., 1994; Kanto et al., 1996; Roth et al., 1995] that different HCV morphological types can be found at different stages of hepatitis C infection. These authors used long-term centrifugation of plasma from infected persons at high speeds in different density gradients followed by the detection of RNA in gradient fractions. Application of the MAB EIA method as proposed in our study has the potential to predict the nature of the HCV particle in circulation and allows for a significantly more simple and quicker method of HCV-core detection. This assay may be useful to further investigate the nature of HCV that circulate in different patient categories. This would undoubtedly be of interest in the study of hepatitis C pathogenetic mechanisms.

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